
The influence of novel bioactive glasses on *in vitro* osteoblast behavior

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Abstract: Implant success requires a direct bond between bone and implant surface. Bioinert implants, such as titanium alloys, are commonly plasma-spray-coated with a bone-bonding, bioactive material such as hydroxyapatite. Such coatings tend to be chemically and topographically inhomogeneous without reproducible properties. A family of bioactive glasses that can be enameled and reliably adheres to titanium alloy has been developed. In this study the cytocompatibility of two of these glass compositions was tested in the as-cast condition. The effects of these glasses on the early and late events of osseous tissue formation *in vitro* were determined with MC3T3-E1.4 mouse osteoblast-like cells. MC3T3-E1.4 cells were cultured on glasses containing 55 and 50 wt % SiO₂, with titanium alloy (Ti6Al4V) and tissue culture polystyrene as controls. Cellular adhesion and

proliferation, and alkaline phosphatase activity were studied over 5 to 15 days in culture. Qualitative and quantitative assays of mineralization were conducted. The osteoblast-like cells showed increased proliferation when grown on a bioactive glass containing 50 wt % silica. However, the adhesion, differentiation and mineralization behavior were similar on both glass compositions used in this study. These bioactive glasses proved to be cytocompatible substrata for osteoblast-like cell culture, and yielded higher cellular proliferation than titanium alloy. © 2004 Wiley Periodicals, Inc. *J Biomed Mater Res* 71A: 242–249, 2004

Key words: bioactive glass; cell–material interactions; bio-compatibility; hard tissue

INTRODUCTION

The long-term success of orthopedic and dental implants depends on osteointegration.¹ A number of factors affecting osteointegration include implant material, design, surface chemistry and topography, quality of the bone, wound healing process, and operator error.² Bioinert materials with suitable mechanical properties, such as stainless steel, titanium, and cobalt chromium alloys (i.e., 316L, Ti6Al4V, and Co-Cr-Mo), are commonly used for making load bearing implants.³ Their retention relies mostly on mechanical interaction. Bioinert implants often become encapsulated in fibrous tissue, which results in the lack, or improper transfer, of stress across the interface,

thereby loosening the implant or fracturing the adjacent bone.⁴

To improve implant–bone bonding, metal alloys are commonly coated with hydroxyapatite (HA) by plasma spray^{5,6} or other techniques.^{7–10} Each technique presents pitfalls that result in partial crystallization and/or poorly controlled macro- and microporosity that affect the reproducibility, stability, and long-term performance of the coatings.¹¹ Metal alloys also can be coated with some bioactive glasses by the plasma spray technique,¹² subject to the same drawbacks. Glasses of bioactive compositions allow the formation of a biological apatite layer, similar to natural bone, on their surfaces *in vitro* when immersed in simulated body fluid (SBF). This is a cell- and protein-free solution reproducing the ionic concentrations of blood plasma.^{13,14} The formation of this layer is thought to be responsible for the bone-bonding characteristics of bioactive glasses.^{15,16} Bioglass®, has found many non-load-bearing applications and its interactions with bone *in vivo*, and with osteoblast cultures *in vitro*, have been extensively studied.^{17–22} It is known that bioactive glasses are not strong enough to

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withstand loads, and attempts to use Bioglass® as an implant coating resulted in unreliable glass-metal bonding.^{3,23}

A family of bioactive glasses has been developed that can be enameled onto Ti6Al4V yielding a reliable coating²³ while retaining bioactivity.²⁴ These glasses have the same phosphate content as Bioglass®, but differ in the amounts of other oxides. They also contain potassium and magnesium oxides, which are absent in Bioglass®, and allow a better match to the coefficient of thermal expansion of Ti6Al4V. An enameled bioactive coating may provide the desired interfacial attachment to bone, extending implant life. More uniform surface texture, better adhesion, and controlled chemical composition of bioactive glass coatings may make them more reproducible and reliable than plasma-sprayed HA. To successfully fabricate functionally graded coatings on Ti6Al4V, the compositional limit for the top layer is about 53 wt % SiO₂. Because the chemical compositions of the glasses in this study differ significantly from Bioglass®, it is logical to test their cytocompatibility. As suggested by ASTM F748-98,²⁵ we first tested the glasses as cast without engaging in the coating fabrication process, to determine whether the glasses contain components that, once leached into solution, may adversely affect osteoblast-like cell activity. It was hypothesized that these glasses do not have a detrimental effect on osteoblast-like cellular behavior and are cytocompatible. The objective of this study was to screen for possible adverse reactions and/or differences in cellular response to the two glass compositions. We assayed cell adhesion, proliferation, differentiation, and mineralization behavior of the mineralizing subclone of the mouse osteoblast-like cell line MC3T3-E1: MC3T3-E1.4 applied to two bioactive glasses and to the control substrates, Ti6Al4V and tissue culture polystyrene.

MATERIALS AND METHODS

Glass preparation

Glasses in the SiO₂-Na₂O-K₂O-CaO-MgO-P₂O₅ system were prepared by mixing the reagents in propanol using a high-speed stirrer for 1 h followed by drying at 80°C for 12 h.²³ The P₂O₅ content was 6 wt % for both glasses, therefore designated 6P followed by their SiO₂ content in weight percent (Table I).

The reagents were SiO₂ (99.5%, Cerac Inc., Milwaukee, WI), CaCO₃ (99.9%, J.T. Baker, Phillipsburg, NJ), MgO (98.6%, J.T. Baker), K₂CO₃ (99%, Allied Chemical, Minneapolis, MN), NaHCO₃ (99.5%, J.T. Baker), and Na₂HPO₄ (99.7%, Allied Chemical, Minneapolis, MN). After drying, the mixture was fired in air in a Pt crucible for 5 h at 1400°C. The melt was cast in a preheated (200°C) graphite mold

TABLE I
Glass Compositions in Weight Percentage

	SiO ₂	Na ₂ O	K ₂ O	CaO	MgO	P ₂ O ₅
6P50	49.8	15.5	4.2	15.6	8.9	6.0
6P55	54.5	12.0	4.0	15.0	8.5	6.0

yielding glass bars ($\approx 2 \times 2 \times 8$ cm) that were annealed at 500°C for 6 h to relieve internal stresses. The annealed bars were subsequently cut perpendicularly to the long axis into square samples ($\approx 2 \times 2 \times 0.2$ cm) with a low speed diamond saw (Isomet, Buehler, Ltd., Lake Bluff, IL). Each sample was metallographically polished with diamond suspension to 1 μ m (Buehler, Ltd.). Samples were then reduced to squares of $1.5 \times 1.5 \times 0.2$ cm by grinding down the sides. The resulting glass squares fit snugly into the wells of 12-well tissue culture polystyrene plates (B-D Falcon, Becton Dickinson, Franklin Lakes, NJ), which prevented the samples from moving and therefore avoided the breakage of the cell layer when the plates were handled for medium replenishment. Cutting and polishing was done in isopropyl alcohol due to the reactivity of the glasses in aqueous solutions. Titanium alloy (Ti6Al4V Goodfellow Ltd., Huntingdon, UK), similarly cut into squares and polished, served as control together with tissue culture polystyrene (TCPS; the bottom of the wells). Prior to sterilization in dry heat at 250°C, the samples were cleaned by sonication in acetone and isopropyl alcohol for 5 min each. Sterile samples were placed in 12-well tissue culture plates under sterile conditions.

Cell culture

MC3T3-E1.4 mouse osteoblast-like cells were obtained from Dr. Tamara N. Alliston (Dr. Rik Derynck's laboratory, University of California, San Francisco). This is a well-characterized mineralizing subclone of the mouse osteoblast-like MC3T3-E1 cell line.²⁶⁻³⁰ Cells were grown in an incubator at 37°C and 5% CO₂ atmosphere, in α -modified Eagle's medium (α -MEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics (penicillin and streptomycin) and passaged every 4 to 5 days. Cells were used between passage 10 and 20. In all experiments cells were plated at an initial density of 50,000 cells/cm²; previous studies showed that this cell density results in optimal expression of osteoblast markers.³⁰

Cell adhesion

Cells (50,000 cells/cm² density) were seeded in 30 μ L aliquots on the center of each sample and control material. Cells were allowed to adhere for 10 min before the wells were gently flooded with the medium (1 mL/well). We consistently used 1 mL medium/well to reduce the variability of glass dissolution which is known to be a function of the solution volume.³¹ Three hours after flooding, the supernatant containing non-adhering cells was removed, and re-

placed with fresh medium. The number of adhering cells was assayed using a commercial kit (CellTiter 96®, Promega Corp., Madison, WI), based on the metabolic activity of living cells [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; MTT] assay]. The MTT solution added to each well was incubated for 4 h at 37°C. After the formation of the crystal formazan product the square samples (glasses and Ti6Al4V) were transferred from the original tissue culture plate and placed into the wells of a new one. This way only the crystals formed on the samples were dissolved. To dissolve the formazan product solutions were added according to the Promega protocol, keeping the ratio of solution volume to sample surface area constant at 2 $\mu\text{L}/\text{mm}^2$. This was done to compensate for the difference in surface available to the cells, because the samples did not cover the bottom of the wells completely. The overnight protocol was chosen to ensure complete solubilization of the dark blue formazan product. The optical density was measured at 570 nm in a spectrophotometer (Spectronic Genesys 5, Spectronics Instruments, Rochester, NY). A calibration curve was determined to convert absorption into number of cells; the relationship was linear. The data was normalized to the sample surface area (cells/cm^2). Experiments were performed in triplicate with $n = 3$ for each material in each experiment.

Cell proliferation

Cell proliferation was assayed according to the following schedule: on day 1, cells were seeded as described above; on day 2, cells were synchronized by serum starvation for 48 h, replacing the medium with a fresh one containing 1% FBS. On day 4, cells were allowed to re-enter the cell cycle by replacing the medium with a fresh one containing 10% FBS. After 24 h, the proliferating cells were assessed using the MTT assay as described above. Experiments were performed in triplicate with $n = 3$ for each material in each experiment.

Alkaline phosphatase

To determine the effect of the glasses on early events of osseous tissue formation *in vitro*, alkaline phosphatase activity, a marker of osteoblast differentiation, in the absence of ascorbic acid (AA) treatment, was analyzed. Cells were seeded as described above, the medium was replaced at 2-day intervals throughout the experiment. Alkaline phosphatase activity was measured in cell layers after 5, 8, 11, and 15 days in culture. At each time point the glasses and Ti6Al4V were transferred to a new tissue culture plate to avoid lysis of cells not on the materials. Cell layers were rinsed twice with phosphate buffer solution (PBS) without calcium or magnesium, incubated with 100 μL sodium dodecyl sulfate solution (0.05% in PBS). Alkaline phosphatase activity was measured, from 10 μL of cell lysate, as released *p*-nitrophenol from a *p*-nitrophenyl phosphate substrate after 15 min of incubation at 37°C (Sigma Diagnostics Procedure No. 104, Sigma Diagnostics Inc., St. Louis, MO). A Sigma Unit of phosphatase activity is the amount of enzyme

that will release 1 μmol *p*-nitrophenol per hour. The optical density was measured at 410 nm in a spectrophotometer. The enzyme activity was expressed as units per milligram of total proteins. Estimation of protein content was carried out using a Bio-Rad Protein assay (BioRad Laboratories, Hercules, CA); optical density was measured at 595 nm in the same spectrophotometer. To determine the effect of the materials on cellular response to AA, experiments at day 3 and 8 were repeated in presence of AA (50 $\mu\text{g}/\text{mL}$ medium) and alkaline phosphatase was measured as described above. Experiments were done at least twice with $n = 3$ per material per experiment.

Mineralization

Alizarin S staining

Cells were seeded as described above. Cells were treated with AA (50 $\mu\text{g}/\text{mL}$ medium) for 10 days and then with AA and inorganic phosphate (NaH_2PO_4 ; 10 mM) for an additional 5 days. Medium was replaced at 2-day intervals throughout the experiment. Cells were washed 3 times with PBS without calcium or magnesium prior to fixation with 100% ethanol for 15 min. Cell layers were stained with alizarin S solution for 30 min, rinsed and photographed under an optical microscope (Olympus BX50, Olympus America Inc., Melville, NY) with a charged couple device camera (DVC 1300C camera, RGB color, DVC Company, Austin, TX). Experiments were performed in triplicate with $n = 3$ for each material in each experiment. To ensure that no artifact staining occurred due to the presence of phosphate in the glass compositions the following control experiments were carried out: (1) samples were prepared as described above, and half the cell layer covering the glass was removed prior to staining and (2) glass as-cast was stained with alizarin S.

Quantitative measurement of inorganic phosphate

To quantify the amount of mineralized matrix produced by the cells on the different materials, cells were seeded and treated with ascorbic acid and inorganic phosphate to induce mineralization as described above. After 15 days in culture, cells were washed 3 times with PBS without calcium or magnesium and the cell layer grown on glasses and Ti6Al4V was scraped off the sample with a rubber policeman and transferred to a new tissue culture plate. This was done to avoid measuring phosphate released from the glasses themselves or from the tissue grown on the tissue culture wells adjacent to the test substrates, because the glass and Ti6Al4V substrates did not cover the bottom of the wells entirely. The mineralized tissue was incubated for 24 h in 15% trichloroacetic acid (TCA). The amount of TCA added was kept constant at 2 $\mu\text{L}/\text{mm}^2$ of sample surface. Fifty microliters of supernatant was then assayed. Inorganic phosphate concentration was determined by spectrophotometry (355 nm) using the method of Heinonen and Lahti.³² The absorbance value in Klett units (KU) (1 KU corresponds to the absorbance of 0.005) was converted into concentration

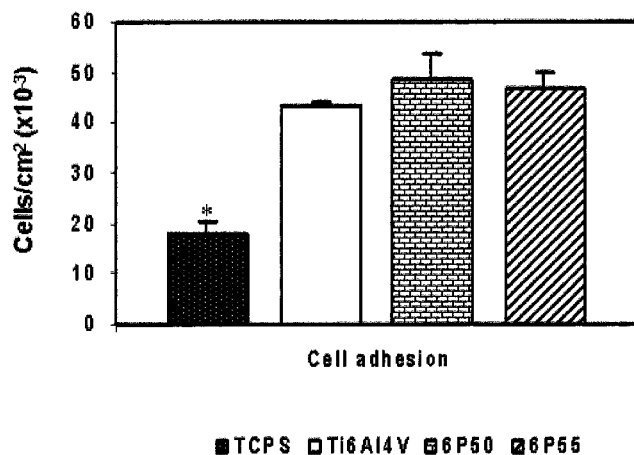


Figure 1. Cell adhesion: cells adhered in lower numbers onto TCPS. *Significantly different with respect to all other materials (ANOVA, SNK $p < .05$).

using a calibration curve made using the same method. Experiments were performed in triplicate with $n = 3$ for each material in each experiment.

Scanning electron microscopy (SEM) analysis

Cell layers cultured and treated for mineralization were prepared for SEM analysis (Topcon ISI SX 40A scanning electron microscope, Milpitas, CA) as follows: Cell layers were dehydrated with a series of graded ethanol solutions, then treated with hexamethyldisilazane (HMDS) and sputter-coated with Au-Pd (200 nm).

RESULTS

Cell adhesion

Figure 1 shows the results from a representative experiment ($n = 3$): the number of viable adhering cells was significantly lower on TCPS with respect to Ti6Al4V and the glasses [ANOVA, Student Newman-Keuls (SNK) $p < .05$].

Cell proliferation

As shown in Figure 2, proliferation was significantly different on each material (ANOVA, SNK $p < .05$). Cells proliferated on the materials in the following order from lowest to highest: TCPS, Ti6Al4V, 6P55, and 6P50.

Alkaline phosphatase

At days 5, 8, 11, and 15 alkaline phosphatase activity was low or undetectable for all samples and controls

with no significant differences. This result was not surprising because the alkaline phosphatase production of this cell line is known to be extremely low in the absence of ascorbic acid (AA) treatment.²⁸ Thus, exposure of the cell line to the test and control materials had no effect on alkaline phosphatase expression in the absence of AA. To determine whether the cell culture was being handled correctly and retained its ability to respond to AA treatment in the presence of the materials we repeated some experiments, on the same substrates, but treated with 50 $\mu\text{g/mL}$ AA. Alkaline phosphatase activity increased significantly after ascorbic acid treatment (with respect to no AA treatment) on each material, but no significant difference between samples and controls was found (data not shown).

Mineralization

Alizarin S staining

Fresh samples were prepared and treated with alizarin S to assess the level of background staining. No staining was found on samples and controls (data not shown). Figure 3 shows alizarin S staining for control samples where part of the cell layer was removed prior to staining. The area of the sample not covered by the cell layer had a pale pink staining. Figure 4 shows alizarin S staining of the mineralizing cell layer on glasses and control materials. Distinct nodular colonies can be distinguished and a morphological difference in the mineral distribution is apparent. Tissue culture polystyrene [Fig. 4(A)] and Ti6Al4V [Fig. 4(B)] showed well-localized areas of mineralization with intense staining. Areas clearly devoid of calcium and unstained also are present. Glasses 6P50 [Fig. 4(C)] and 6P55 [Fig. 4(D)] appear to be mineralizing differ-

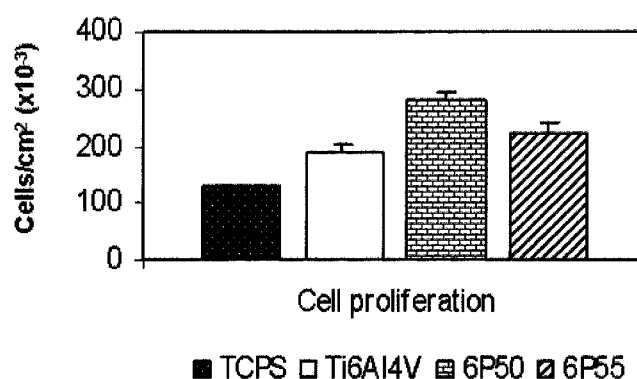


Figure 2. Cell proliferation: after 5 days in culture MC3T3-E1.4 mouse osteoblast-like cells proliferated significantly differently on all materials. Lowest proliferation occurred on TCPS followed by Ti6Al4V, glass 6P55, and glass 6P50 (ANOVA, SNK $p < .05$).

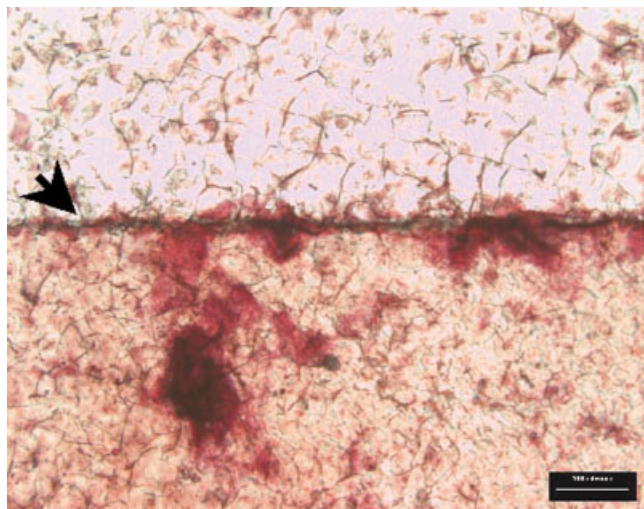


Figure 3. Mineralization, control experiment: tissue grown onto glass 6P50 was cut (black arrow) prior to staining with alizarin S. The tissue stained for alizarin S (area below the arrow), area devoid of tissue shows pale pink staining (area above the arrow). Similar results were obtained with glass 6P55. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

ently, as indicated by the more extensive and less intense staining patterns. Scanning electron microscopy analysis (Fig. 5) revealed areas with the morphology of mineral nodules and abundant collagen-like fibers. Three-dimensional structures attributed to bone nodules are indicated by the black arrowheads in Figure 5 (A, tissue culture polystyrene; B, Ti6Al4V; C, 6P55; D, 6P50). On all materials a collagen-like extracellular matrix was present, but it appeared more evident on the glasses [Fig. 5(C,D,E), large black arrows].

Quantitative measurement of inorganic phosphate

Figure 6 shows the concentration of inorganic phosphate released from the mineralizing cell layer. Significantly higher concentration of inorganic phosphate was released from the cell layer grown onto TCPS (ANOVA, SNK $p < .05$).

DISCUSSION

The ultimate goal is to fabricate a functionally graded coating on titanium alloy implants, yielding a

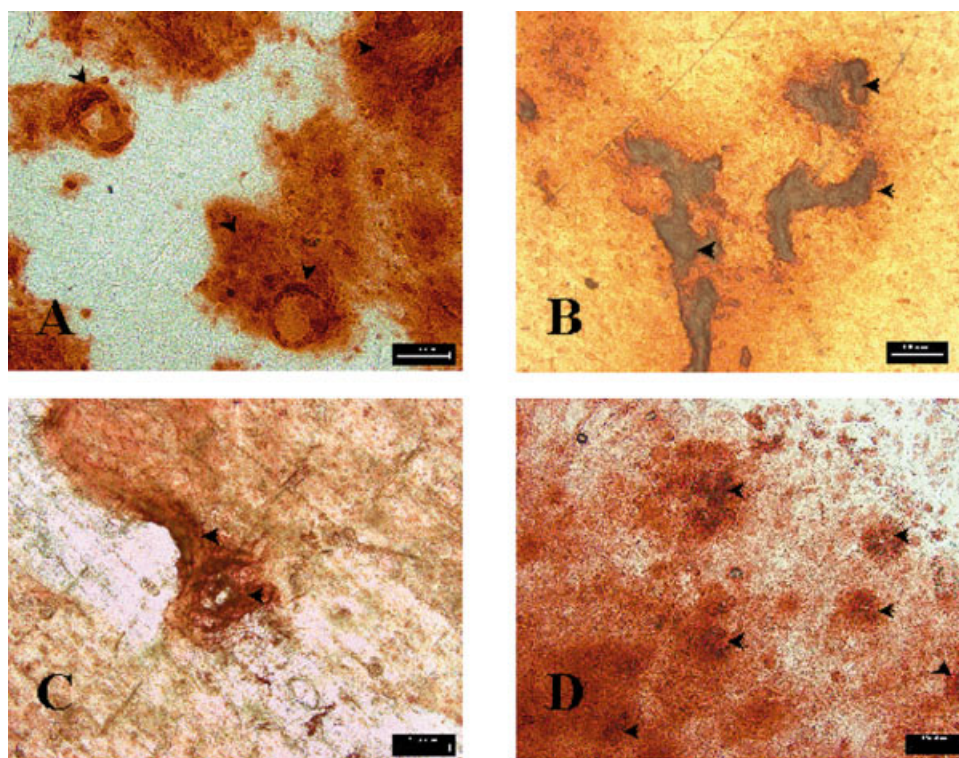


Figure 4. Mineralization by alizarin S staining: mineralizing bone nodules showed a dark red staining (black arrowheads). The nodules appeared to be round in shape on tissue culture polystyrene (A) and on the glasses 6P55 (C) and 6P50 (D). On Ti6Al4V (B) mineralizing nodules had a more irregular shape. The staining was overall more intense and well defined on tissue culture polystyrene (A) and Ti6Al4V (B), where non-mineralizing, unstained areas are also clearly visible. Note that Ti6Al4V is not transparent and the reflected light confers a yellowish color to the unstained areas. On glasses 6P55 (C) and 6P50 (D) mineralization appeared to be more diffuse and less intense; non-mineralizing areas are not well defined. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

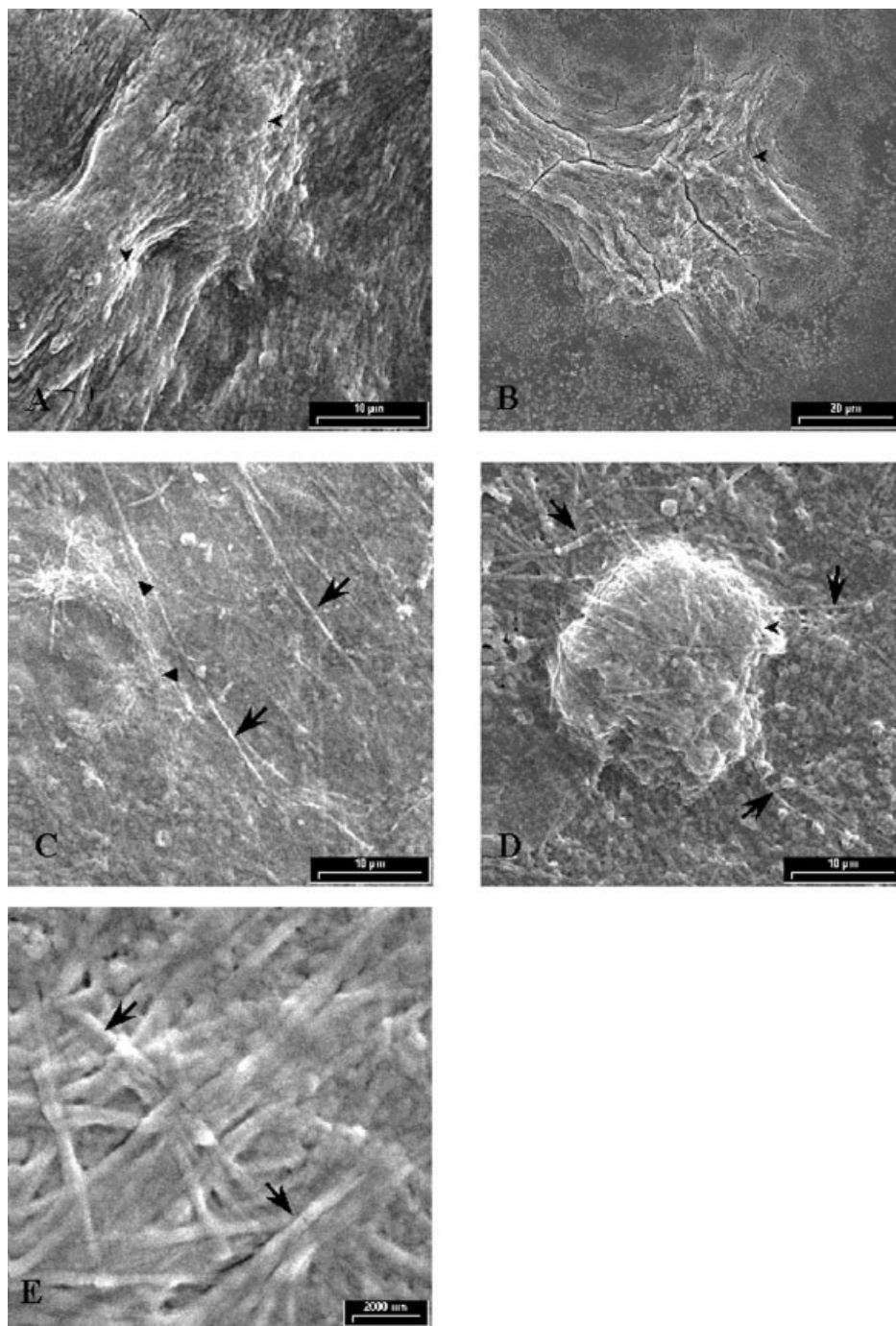


Figure 5. Scanning electron microscopic analysis of mineralized areas: bone nodules of different size were observed on all materials (black arrowheads). The nodules appeared rounded on tissue culture polystyrene (A), and on glasses 6P55 and 6P50 (C and D, respectively), while on Ti6Al4V (B) had a more irregular shape. The bone-like matrix contained collagen-like fibers which was detectable on all materials, but on the glasses was more evident even at low magnification (large black arrows). Representative collagen matrix on glass 6P50 is shown in (E).

reproducible and reliable bioactive surface. The characterization of bioactive glasses for this purpose has important implications in the development of such new implants and possibly in skeletal reconstruction. This is the first time that the *in vitro* biocompatibility of these bioactive glasses has been evaluated with osteoblast-like cells. The MC3T3-E1 cell line is known to be

phenotypically heterogeneous due to its mesenchymal origin and multiple passaging.²⁷ For this reason we chose its subclone, the MC3T3-E1.4, which is well characterized and has the ability to form a mineralized bone matrix *in vitro*.^{26,28–30} Bioactive glasses should support osteoblast proliferation and differentiation. Lack of cellular adhesion on these materials indicates

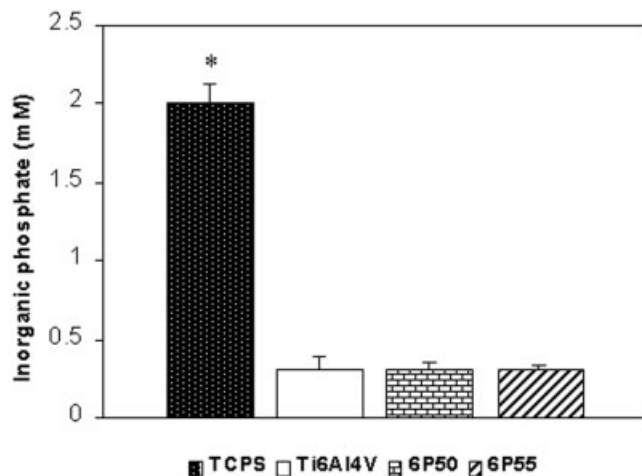


Figure 6. Quantitative mineralization: mineralizing tissue grown onto TCPS released a significantly higher concentration on inorganic phosphate respect to mineralizing tissue grown onto Ti6Al4V and bioactive glasses. * $p < .05$.

cytotoxicity because these cells require adhesion for survival. Three hours after seeding, mouse osteoblast-like cells adhered on all samples and controls. Bioactive glasses, by means of their reactivity in aqueous solutions, produce a silica gel layer on their surfaces¹⁷ which could affect cell adhesion. Cells could be passively stuck on the glass surface but not be viable. To measure cell adhesion we chose the MTT assay, which accounts only for viable cells. The data indicate that MC3T3-E1.4 osteoblast-like cells adhered on tissue culture polystyrene in lower numbers compared to bioactive glasses containing 50 and 55 wt % silica and to titanium alloy. At day 5 proliferation was significantly different on each material. The lowest proliferation, on TCPS, could be due to the lower initial number of adhering cells. Differences in proliferation on the other materials are probably due to the intrinsic properties of the materials because there was no initial difference in the number of adhering cells. MC3T3-E1.4 cells are known to respond to AA treatment by increasing alkaline phosphatase activity. To identify possible changes in alkaline phosphatase activity due exclusively to the bioactive glasses, alkaline phosphatase activity was measured without AA treatment. The natural low alkaline phosphatase activity was not significantly altered by any of the materials. Control experiments were conducted to determine whether the glasses impaired cellular response to AA treatment. Alkaline phosphatase increased in response to AA treatment in the presence of the materials but no significant difference between materials was observed. Thus, bioactive glasses in this study did not appear to perturb osteoblast-like cell alkaline phosphatase activity. Cultures maintained for 15 days in α MEM under mineralizing conditions differentiated, forming three-dimensional nodular colonies. Alizarin S staining and

microscopic analysis indicated that these three-dimensional nodules consisted of aggregated cells embedded in the mineralizing extracellular matrix. Bioactive glasses allowed a more diffuse mineralization pattern, while titanium alloy and tissue culture polystyrene presented localized mineralized areas. Microscopic analysis revealed the presence of abundant extracellular matrix (fibrous in nature), probably collagen that is necessary for mineralization.^{33,34} The collagen organization in the osteoblastic extracellular matrix can be affected by different calcium-phosphate-based bioactive substrates.³⁵ The surface reactivity of the bioactive glasses may play a role in the way collagen is distributed, yielding a uniform and less intense mineralization pattern. Alizarin S, which binds to calcium,³⁶ could have yielded false-positive results for two main reasons: (1) the glasses contain calcium and (2) in aqueous solutions, these glasses react to form a superficial layer of calcium-containing mineral. We eliminated these possibilities by testing for background staining of the glass alone, prior to immersion in any fluid, which yielded no staining. Secondly, mineralizing tissue grown on samples was partially removed prior to staining. After Alizarin S treatment the exposed glass did not stain significantly. Therefore, the stain seen on the glasses was a result of mineralization and not a result of false staining of calcium components of the glasses. We then quantified the mineral formation because the Alizarin S staining could not be quantified due to different optical properties of the glasses compared to the alloy.

The quantity of mineralized tissue grown on the glasses and titanium alloy contained similar amounts of inorganic phosphate, while mineralization was significantly higher on TCPS. The results of this work suggest that these bioactive glasses have significant potential for use as coatings, and have no deleterious effects on an osteoblast-like cell function as studied here. Further quantitative analysis of markers of osteoblast differentiation may reveal subtle differences in the effect of bioactive glass coatings on cellular behavior. Future research will involve the fabrication of functionally graded enameled coatings with a bioactive surface containing about 55 wt % silica and their detailed analysis.

CONCLUSIONS

The cytocompatibility of bioactive glasses, as cast, containing 50 and 55 wt % SiO_2 , was tested using the mouse MC3T3-E1.4 osteoblastic cell line as an *in vitro* bone model. We found significantly higher cell adhesion onto bioactive glasses and Ti6Al4V with respect to tissue culture polystyrene. Proliferation was significantly different on all materials: highest on the glass

containing 50 wt % silica, followed by the glass containing 55 wt % silica, Ti6Al4V, and lowest on TCPS. Alkaline phosphatase activity was not affected by the glasses or control materials. Mineralization occurred on all materials with morphological differences. The mineralizing tissue grown onto bioactive glasses and titanium alloy contained similar amounts of inorganic phosphate. The results suggest that the bioactive glasses studied here do not have any significant negative effect on osteoblast-like cell function and are cytocompatible.

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